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Neonatal Rat Forebrain Anterior Subventricular Zone Do Not Require GFAP-Positive Astrocytes

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A prolific neuronal progenitor cell population in the anterior portion of the neonatal rat forebrain subventricular zone, the SVZa, is specialized for the production of olfactory bulb interneurons. At all ages, SVZa-derived cells traverse a tangential migratory pathway, the rostral migratory stream (RMS), while en route to the olfactory bulb. Unlike other neuronal progenitor cells of the forebrain, migrating progeny of SVZa progenitors express neuronal-specific proteins and continue to divide into adulthood. Recent studies indicate that in the adult, migrating SVZa-derived cells are ensheathed by astrocytes, although the function of these astrocytes has not been determined. To explore the possible role(s) of astrocytes in the rat SVZa and RMS, we examined the expression of astroglial-specific genes in the postnatal SVZa and RMS using RT-PCR, *in situ* hybridization, and immunohistochemistry during (Postnatal Days 1–10) and after the period of peak olfactory bulb interneuron generation. We also examined the expression of neuronal-specific genes throughout the rostral-caudal extent of the postnatal subventricular zone to determine if differential cell type-specific gene expression could distinguish the neurogenic SVZa as a region distinct from the remainder of the SVZ. We found little to no astrocyte-specific gene expression in the P0-P7 SVZa, although the neuron-specific isoforms of tubulin (Tα1 and β-III tubulin) were expressed abundantly in the SVZa and RMS. In contrast, astrocyte-specific genes were strongly expressed in the SVZ posterior to the SVZa. GFAP expressions begins to appear in some restricted areas of the rostral migratory stream after the first postnatal week. These data suggest that astroglia are not involved in the generation or migration of most olfactory bulb interneurons. Moreover, the scarcity of glial markers in the neonatal SVZa indicates that the forebrain subventricular zone includes a distinct neurogenic anterior region containing predominantly committed neuronal progenitor cells.

Key Words: **development; forebrain; neurogenesis; olfactory bulb; progenitor cell.**

INTRODUCTION

The postnatal subventricular zone (SVZ) surrounding the lateral ventricles has long been recognized as a source of forebrain astrocytes and oligodendrocytes (Privat, 1975; LeVine and Goldman, 1988; Vaysse and Goldman, 1990; Levison *et al.*, 1993; Levison and Goldman, 1993). Nevertheless, the complexity of precursor cell types that compose the SVZ is still not fully understood. It is not clear to what extent the SVZ can be subdivided into distinct regions along the anterior-posterior axis of the forebrain according to the types of cells that are generated at specific locations (Levison and Goldman, 1993; Luskin *et al.*, 1993). While much of the forebrain SVZ overlies the lateral ventricle and lies below the corpus callosum, a specialized arm extends rostrally to form the subependymal zone in the middle of the olfactory bulb (see Fig. 1) (for the purpose of this report, *subventricular zone* will be used interchangeably with *subependymal zone*). Previous studies suggested that this rostral extension of the rat postnatal SVZ contained progenitor cells for olfactory bulb interneurons (Altman, 1969) and apparently did not contain astrocytes as evidenced by an absence of tangentially oriented glial fibrillary acidic protein (GFAP) immunoreactive cells in this region (Kishi

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et al., 1990). Subsequently, lineage tracing studies demonstrated that in the neonatal brain of rodents, an essentially pure population of neuronal progenitor cells is situated in the anterior extent of the SVZ, referred to as the SVZa (Luskin, 1993; Zigova *et al.*, 1996).

Throughout life SVZa-derived cells migrate several millimeters from the SVZa to the olfactory bulb along a tangential pathway known as the rostral migratory stream (RMS) and ultimately differentiate into periglomerular and granule cell neurons (Kishi, 1987; Luskin, 1993; Lois and Alvarez-Buylla, 1994; Menezes *et al.*, 1995). In the neonate, there is little evidence that glial guidance contributes to the migration of SVZa-derived cells in the RMS. Kishi *et al.* (1990) reported that GFAP immunoreactivity in the early postnatal SVZa and RMS is sparse and that the few GFAP-positive fibers that are detected are oriented perpendicular to the axis of migration of SVZa-derived cells. In the adult RMS, in contrast to the neonate, closely associated chains of migrating neurons are ensheathed by a meshwork of specialized GFAP-positive astrocytes (Lois and Alvarez-Buylla, 1994; Lois *et al.*, 1996). More recent studies suggest that GFAP-positive astrocytes promote adult SVZa neurogenesis (Lim and Alvarez-Buylla, 1999), and that in the adult the stem cells within the SVZa are the GFAP-positive astrocytes (Doetsch *et al.*, 1999). It is not yet clear to what extent GFAP-positive cells are required for adult SVZa cell migration or neurogenesis. Although the close physical interaction between the adult SVZa-derived cells and the astrocytes invites speculation that the astrocytes might guide the stereotypic migration of SVZa-derived cells, there is no evidence that these GFAP-positive ensheathing cells are necessary to direct SVZa cell migration in the RMS. Indeed, when explants of the region of the SVZa are cultured in a three-dimensional matrix, cells with a neuronal phenotype migrate rapidly away from the explant in close association with each other, in the absence of GFAP-positive astrocytes or other glia (Wichterle *et al.*, 1997). This is also consistent with our observation that dissociated neonatal SVZa cells in primary culture uniformly express neuron-specific β-III tubulin and do not express GFAP (Luskin *et al.*, 1997). Nevertheless, the potential importance of GFAP-positive astrocytes to adult SVZa cell production and/or migration raises the question of whether astrocytes play similar roles in the neonatal SVZa, during the period of peak production of olfactory bulb interneurons from this region.

SVZa cells, unlike most other populations of neural progenitors, continue to divide throughout life (Altman, 1969; Lois and Alvarez-Buylla, 1994), providing a constant source of new interneurons for the olfactory bulb. Surprisingly, the actively migrating SVZa-derived cells undergo cell division while they are in the RMS. These migrating cells express a neuronal phenotype normally restricted to postmitotic neurons (Menezes *et al.*, 1995). *In vivo* and *in vitro* lineage studies indicate that glia originate predominately in the more posterior region of the SVZ (Luskin and McDermott, 1994) although some cortical neurons may also arise from the posterior SVZ (Levison and Goldman, 1997). These observations suggest that the anterior and posterior portions of the subventricular zone may be fundamentally different and prompted us to compare the expression of cell-type specific genes in these two regions during postnatal development.

Our studies demonstrate that SVZa cells express markers for differentiating neurons, but do not express astrocytic markers, at least for the first week of postnatal rat development. On the other hand, astrocyte-specific markers are readily detectable in the posterior SVZ of the newborn rat. These data support the suggestion that the SVZa progenitor cell population is distinct from the progenitors of the posterior subventricular zone. In addition, the absence of astrocyte-specific mRNA and protein expression in the SVZa, and to a large extent the RMS, indicates that astroglia are not required for either SVZa neurogenesis or migration during the first postnatal week which coincides with the period of peak production of SVZa-derived olfactory bulb interneurons.

MATERIALS AND METHODS

Microdissection

The procedures used to obtain SVZa neuronal progenitor cells, ventricular zone (VZ) neuroepithelium and the posterior subventricular zone (SVZp) from newborn and perinatal rat pups have been previously described (Zigova *et al.*, 1996; Luskin *et al.*, 1997). Our convention was to designate the day on which a vaginal plug was detected as Embryonic Day 0 (E0); for rats, birth usually occurs at E22, also considered to be Postnatal Day 0 (P0). For SVZa and SVZp dissections, P0-P1 rat pups were anesthetized by hypothermia and decapitated. The head was immediately immersed in ice-cold Ham's F-10 nutrient mixture. The brain was removed, transferred to fresh media, and bisected at the sagittal midline. A parasagittal section approximately 2 mm thick was taken from each half of the brain and the meninges were carefully removed. The SVZa was identified by its position at the anterior-most lateral wall of the lateral ventricle and by its slightly darker, more transparent appearance compared with the overlying corpus callosum. In order to avoid including the striatum and corpus callosum, the most dorsal and central parts of the SVZa were not included. For the SVZp dissection, the caudal part of the subventricular zone was isolated (Fig. 1A). Both regions were excised with a microknife.

Neocortical VZ tissue was harvested from embryos of timed-pregnant rats at the onset of cortical neurogenesis, coinciding with E13. Pregnant dams were anesthetized with chloral hydrate (350 mg/kg, ip) and a small incision was made in the lower peritoneal cavity to allow access to the uterine horns. Embryos were removed one at a time from the anesthetized mother and placed in ice-cold Ham's F-10 as described above. Brains were removed and sagittally bisected at the midline, and the meninges were completely removed. The VZ was dissected away from the dorsal-most lateral part of the telencephalon to obtain a small ($\sim$0.5 mm^3) tissue segment (Zigova *et al.*, 1996). Anesthetized mothers were sacrificed after the removal of the embryos.

TABLE 1
Oligonucleotide Primers Employed in This Study

	Forward primer 5′ → 3′	Reverse primer 5′ → 3′	Reference
GAPDH	ATCACCATCTTCCAGGAGCG	TAGGAACACGGAAGGCCATG	McKinney and Robbins (1992)
GFAP	CTCAATGCTGGCTTCAAGGAGA	GACGCAGCGTCTGTGAGGTC	Lewis *et al.* (1984)
Glutamine synthetase	GGTATGCGAAGACTTTGGG	GCCAGTTTCGTTGAGGAGACAC	Mearow *et al.* (1989)
Neuron-specific α-I tubulin	CCAAAGATGTCAATGCTGCCA	CATCAGTGAAGTGGACGGCTC	Ginzburg *et al.* (1986)
Neuron-specific β-III tubulin	TGCGTGTGTACAGGTGAATGC	AGGCTGCATAGTCATTTCCAAG	Jiang and Oblinger (1992)
Nestin	GGAGTGTCGCTTAGAGGTGC	CAGCAGAGTCCTGTATGTAGCC	Lendahl *et al.* (1990)

RNA Preparation

Microdissected tissue for preparation of RNA was immediately frozen by transfer to an RNase-free microfuge tube held on dry ice. RNA was prepared from the tissue using acid phenol extraction as described by Chomczynski and Sacchi (1987). Possible DNA contamination was removed by treatment with RQ1 RNase-free DNase (Promega) as described by the supplier. RNA quantity and quality were assessed by spectrophotometry and denaturing agarose gel electrophoresis.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

To detect specific gene expression in microdissected neural progenitor cell regions, a two-step RT-PCR was employed following a modification of the protocol described by Freeman *et al.* (1994). Briefly, 1 μg of total RNA combined with 10 μM random hexamer deoxyoligonucleotide primers (Promega) was heated to 70°C for 5 min and then quenched on ice. This RNA/primer mixture was placed at 42°C and the reverse transcription reaction mixture [10 mM dithiothreitol, 1 mM each of dATP, dCTP, dGTP, and dTTP, 25 units RNase inhibitor (Promega), and 60 units SuperScript reverse transcriptase (Gibco/BRL)], also held at 42°C, was added to the RNA/primer mixture in a final reaction volume of 25 μl. After 60 min of cDNA synthesis, 75 μl H_2O was added and the reaction terminated by boiling for ten min. One to ten percent of the cDNA mixture was subjected to PCR amplification with primer sets specific to the gene of interest. Twenty to thirty PCR cycles were performed to achieve amplification of the mRNA of interest with typical cycle parameters of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s. For amplification of the relatively invariant "housekeeping" gene GAPDH, PCRs were sampled early in the amplification process so that similar amounts of amplicon indicate that approximately equal quantities of cDNA were included as template for the PCRs. Similarly, PCRs for cell type-specific cDNA were sampled early, prior to the plateau of specific reaction product.

The absence of contaminating genomic DNA in the RT-PCR analysis was demonstrated with parallel RT reactions from the same RNA in which the reverse transcriptase was omitted (Fig. 1B). The specificity of the primer sets employed was confirmed by direct sequencing of the PCR products. The sequences of the primers and PCR products for all genes analyzed in this study are unique to the gene of interest. Primer sequences for each gene are provided in Table 1. Primers were designed from cDNA sequences retrieved from the GenBank DNA sequence databases except for the β-III tubulin primers. The primers for this cDNA were designed from our DNA sequence data obtained from a β-III tubulin cDNA clone generously provided by Dr. M. Oblinger (Jiang and Oblinger, 1992). For RT-PCR analysis, multiple dissections (three or more) of SVZa and SVZp were tested independently.

In Situ Hybridization and Immunohistochemistry

RT-PCR products unique to GFAP, GS, and Tα1 (see Table 1) were subcloned in pGEM-T (Promega) to enable synthesis of single-stranded digoxigenin-labeled RNA probes using dig-UTP RNA labeling mix (Boehringer-Mannheim) and either T7 or SP6 RNA polymerase as described by the supplier. The *in situ* hybridization protocol was modified from Schaeren-Wiemers and Gerfin-Moser (1993) and Ma *et al.* (1997). At least three rats were examined for each postnatal (P) age; P0/P1, P7, P14, and P21 animals were anesthetized by ether and were perfused transcardially with 4% paraformaldehyde in PBS. Cryostat cut brain sections (10 μm) were collected on Superfrost glass slides (Fisher), permeabilized with proteinase K (1 μg/ml in 0.1 M Tris-HCl and 50 mM EDTA, pH 8, 30 min at 37°C), and acetylated (in 0.25%, v/v, acetic anhydride in 0.1 M triethanolamine-HCl). Tissue sections were prehybridized for 4 h at 50°C in hybridization buffer [50% formamide, 0.1 M NaCl, 20 mM Tris (pH 8.0), 5 mM EDTA, 1X Denhardt's solution, 10% dextran sulfate, and 10 mM dithiothreitol (DTT)] and hybridized at 68°C overnight in the same buffer with 1 μg/ml added digoxigenin-labeled probe. The sections were washed for 10 min in 1X SSC (0.15 M NaCl, 15 mM $NaC_6H_5O_7$, pH 7.0) and 10 min with 1.5X SSC at 60°C, followed by two washes at 37°C in 2X SSC for 20 min each. Sections were digested with 0.1 μg/ml RNase A in 2X SSC at 37°C for 30 min, followed with two 10-min washes in 2X SSC at room temperature and two 30-min washes in 0.2X SSC at 60°C. Following equilibration in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5, sections were blocked for 1 h at room temperature in blocking reagent (Boehringer Mannheim) and hybridization was detected immunologically as described by the supplier of the anti-digoxigenin antibody (Boehringer-Mannheim). Sections were incubated with anti-digoxigenin Fab fragments (1:5000) in blocking buffer, washed for 1 h in maleic acid buffer, and developed 4 h to overnight in color development buffer (0.1 M Tris, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$, 0.1% Tween 20, 10% polyvinyl alcohol, 0.315 mg/ml nitroblue tetrozolium, 0.175 mg/ml bromo-chloro-indoyl-phosphate). The color development reaction was terminated in neutralizing buffer (10 mM Tris, pH 5, 1 mM EDTA). Some sections were counterstained with nuclear fast red (Vector Labs) for 5 min and rinsed twice in water for 5 min. Slides were dehydrated through an alcohol series, coverslipped in Permount (Sigma), and

viewed and photographed with a Zeiss Axiophot microscope and IP Lab Spectrum Image Analysis System.

Immunohistochemical detection of GFAP and β-III tubulin was performed on the brains of at least three rats for each age (P0/P1, P7, P14, and P21) examined as we have previously described (Menezes *et al.*, 1995; Zigova *et al.*, 1996; Luskin *et al.*, 1997; Smith and Luskin, 1998). Sections prepared as described above for *in situ* hybridization were pretreated with blocking serum for 60 min and then incubated overnight at 4°C in primary antibody [the TuJ1 monoclonal antibody recognizing β-III tubulin (a gift from Dr. A. Frankfurter) was diluted 1:500 in blocking serum; the anti-GFAP monoclonal antibody was diluted 1:200 in blocking serum (Sigma)]. Primary antibody binding was detected following incubation with fluorescently labeled secondary antibody (rhodamine-conjugated goat anti-rabbit IgG or fluorescein-conjugated goat anti-mouse IgG; diluted 1:200 in blocking serum; Jackson ImmunoResearch) for 1 h at room temperature. Cell nuclei were identified with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) nuclear stain diluted 1:1000 is Tris-buffered saline prior to coverslipping the sections with Vectashield (Vector Labs). To demonstrate specificity, in separate control sections the primary or secondary antibodies were omitted. The sections were viewed with a Zeiss Axiophot fluorescence microscope. In some cases, rhodamine fluorescence was photographed with 35-mm film and appears yellow. In other cases, fluorescence images of the same secondary antibody were digitized directly on the microscope and appear red. All images were captured with the IP Lab Image Analysis System.

RESULTS

The rat SVZa can be distinguished *in vivo* as an expansion of the subventricular zone along the anterior dorsal wall of the lateral ventricle (Fig. 1A; Luskin, 1993). Previous studies have demonstrated that this region can also be distinguished by the phenotypic properties of the resident cells (Menezes *et al.*, 1995; Luskin *et al.*, 1997). The region of the SVZa is a highly proliferative population in the first two postnatal weeks (Altmann, 1969; Bayer, 1983) but despite their capacity for cell division, SVZa cells concurrently express neuron-specific β-III tubulin and microtubule-associated protein 2 (MAP2), markers elsewhere confined to postmitotic neurons (Menezes *et al.*, 1995; Pencea and Luskin, unpublished observation). Unlike other forebrain neurons which withdraw from the cell cycle before the initiation of migration (Rakic, 1990), SVZa-derived cells with a neuronal identity continue to divide in the RMS while *en route* to the olfactory bulb (Fig. 1A) (Kishi, 1987; Menezes *et al.*, 1995; Smith and Luskin, 1998). Furthermore, whereas most forebrain neurons migrate in close association with radial glial fibers (Rakic, 1990), SVZa-derived neurons apparently do not. However, although all SVZa-derived cells migrate to the olfactory bulb and differentiate into neurons, suggesting that the SVZa is a pure neuronal progenitor population, the presence of astrocytes in the early postnatal SVZa and RMS has not been ruled out. Kishi and colleagues (1990) reported sparse, radially oriented GFAP immunoreactivity in the RMS and SVZa at early postnatal ages, but GFAP mRNA was not examined and the relative levels of GFAP immunoreactivity in the SVZa compared to the rest of the SVZ were not described. Furthermore, elaborate GFAP-positive astrocytic arrays have recently been described in the adult RMS (Lois

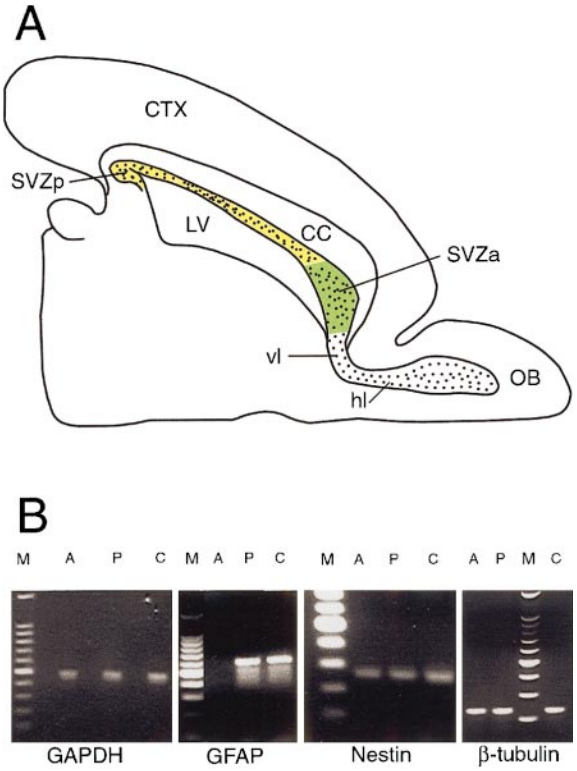


FIG. 1. Differential cell-type specific gene expression by neonatal neural progenitor cell populations. (A) Schematic drawing of a parasagittal section from the P1 rat brain. The stippled region indicates the subependymal zone lining the telencephalic lateral ventricle (LV). The anterior subventricular zone (SVZa, green) is situated along the anterior-most lateral wall of the ventricle. The posterior subventricular zone (SVZp, yellow) corresponds to the subependymal zone of the lateral ventricle that is posterior to the SVZa. For RT-PCR analysis, only the caudal part of the SVZp was microdissected. Abbreviations: CC, corpus callosum; CTX, cerebral cortex; hl, horizontal limb of the rostral migratory stream; OB, olfactory bulb; vl, vertical limb of the rostral migratory stream. (B) RT-PCR amplification of selected genes expressed by astrocytes (GFAP), undifferentiated progenitor cells (nestin), neurons (neuron-specific β-III tubulin) and of a housekeeping gene (GAPDH) from neonatal (P0) SVZa (A), SVZp (P), and frontal cortex (C). Equal amounts of cDNA prepared from each region were subjected to PCR amplification with primers specific for the selected genes (Table 1). Companion control reactions with the same RNA template but without reverse transcriptase are included in alternate lanes in the GAPDH panel. The absence of product in these lanes indicates that genomic DNA contamination does not contribute to the PCR products. One hundred-basepair molecular weight standards (M) are included in each panel. The microdissected SVZa expresses the mRNA for β-III tubulin and nestin but not GFAP. In contrast, expression of β-III tubulin, nestin, and GFAP is detected in the microdissected SVZp and cerebral cortex.

and Alvarez-Buylla, 1994; Lois *et al.*, 1996), raising the question of when the astrocytic network appears in this pathway.

RT-PCR Analysis of Subventricular Zone Gene Expression

Reverse transcriptase-polymerase chain reaction was used to determine if the unique properties of SVZa neuronal progenitor cells could be distinguished at the level of differential mRNA expression. The expression of cell type-specific genes in microdissected rat SVZa tissue was compared to tissue from microdissected posterior-most subventricular zone, a region including glial progenitor cells (Levine and Goldman, 1988; Levison and Goldman, 1993; Luskin and McDermott, 1994). As expected, the expression of the mRNA encoding the astrocyte-specific protein GFAP is readily detected in the SVZp at P0/P1. This message, however, is not detected in RNA isolated from the microdissected P0 SVZa. Conversely, the mRNA for the neuron-specific β-III tubulin and the mRNA encoding the neuroepithelial-specific intermediate filament protein nestin are readily detected in the SVZa and in the SVZp at P0/P1 (Fig. 1B). This RT-PCR analysis suggests that the newborn SVZ can be subdivided into a posterior region expressing astroglial-specific genes, and an anterior region expressing early neuronal- but not astroglial-specific mRNAs. Expression of mRNAs specific to neurons but not astrocytes supports the suggestion that the neonatal SVZa is predominantly neurogenic.

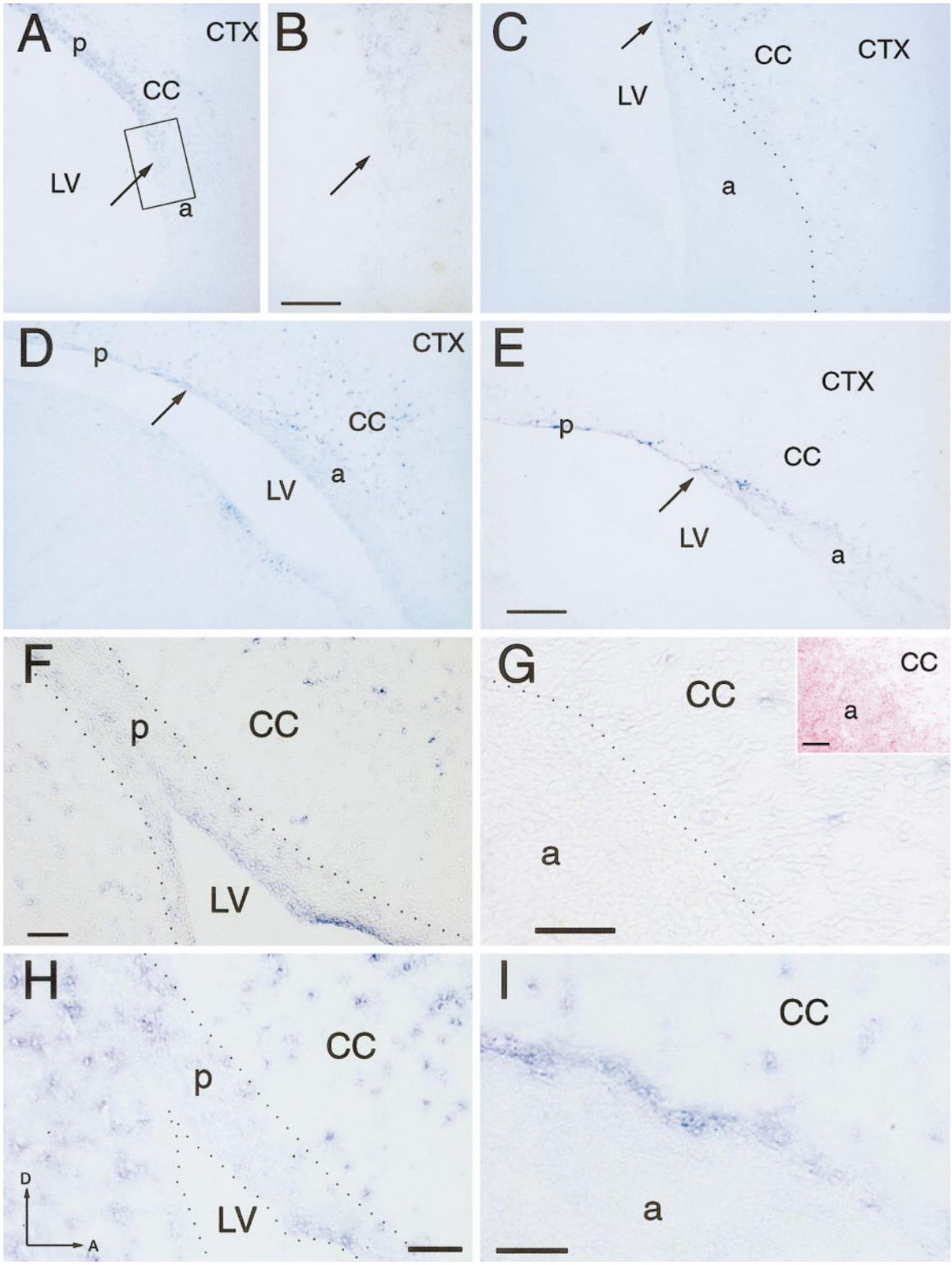
In situ Localization of Cell-Type-Specific Gene Expression in the SVZ

To provide independent verification of our RT-PCR results and to determine the cellular distribution of gene expression along the anterior-posterior axis of the rat SVZ at different postnatal ages, we have localized the expression of astroglial-specific [GFAP and glutamine synthetase (GS)] and neuron-specific [α-1 tubulin (Tα1)] mRNAs with *in situ* hybridization (Figs. 2 and 3). Expression of astroglial-specific mRNAs along the rostral-caudal extent of the SVZ is demonstrated in Fig. 2. The SVZa is essentially devoid of GFAP mRNA at P0 (Figs. 2A and 2B) as indicated by the transition from the strong GFAP mRNA hybridization in the SVZp to weak hybridization in the transition zone (Fig. 2B). The scarcity of GFAP-expressing cells in the SVZa is consistent with cell lineage tracing data which indicate that the SVZa gives rise exclusively to neurons (Luskin, 1993). Another explanation for the absence of GFAP mRNA in the anterior P1 SVZa is that this region may be relatively immature compared to the posterior subventricular zone and thereby not yet competent to produce astroglia expressing GFAP. To address this latter possibility we have examined GFAP mRNA expression with *in situ* hybridization at P7, P14, and P23 (Figs. 2C, 2D, and 2E). GFAP mRNA was essentially undetectable in the SVZa at p7 and only a few GFAP-positive cells were found P14. This is in contrast to the SVZp in which GFAP mRNA is readily detected at all ages examined, reflecting ongoing postnatal gliogenesis (Fig. 2 and data not shown).

The expression of the gene encoding astrocyte-specific glutamine synthetase was determined at P0 and P7 to further address the possibility that less mature, GFAP-negative astrocytes might contribute to the cellular composition of the neonatal SVZa. As for GFAP at both P0/P1 and P7, expression of GS mRNA was detected in the SVZp (Figs. 2F and 2H), but this mRNA was essentially undetectable within the SVZa at these ages (Figs. 2G and 2I). A similar section of P0 SVZa counterstained to demonstrate cellular nuclei indicates the densely packed GS-negative SVZa cells (Fig. 2G, inset). Ependymal cells lining the lateral ventricle uniformly express GS at all ages examined (Figs. 2F and 2H), in agreement with previous reports (Mearow *et al.*, 1989). Otherwise, the distribution of GS-positive cells in the neonatal SVZa is strikingly similar to that of GFAP-positive cells. Thus, it is unlikely that the absence of GFAP mRNA expression in the neonatal SVZa reflects an immature astroglial population since GS mRNA is also not detected and this gene has been reported to be expressed by astrocytes earlier than GFAP (Akimoto *et al.*, 1993). The lack of expression of these astrocyte-specific genes in the neonatal SVZa argues strongly that this cell type does not contribute to the SVZa olfactory bulb interneuron progenitor cell population, at least for the first postnatal week.

In contrast to GFAP, the neuron-specific Tα1 mRNA is readily detected in the neonatal SVZa, throughout the RMS,

FIG. 2. The absence of astrocyte-specific mRNA expression in the neonatal SVZa. (A–E) A digoxygenin-labeled antisense GFAP probe was hybridized to representative 10-μm parasagittal sections from the forebrain of P0 (A, B), P7 (C), P14 (D), and P21 (E) animals. In each photomicrograph the arrow approximates the transition between the SVZa and the SVZp. The black box in A is shown at higher magnification in B to demonstrate the transition from SVZp to SVZa. The boundary between the SVZa and corpus callosum at P7 (C) is demarcated by the dotted line. The P0 (A) and P7 (C) SVZa does not express detectable GFAP mRNA. Even at P0, the transition from the GFAP-positive SVZp to the GFAP-negative SVZa is apparent (A, B). The SVZa at P14 (D) and P21 (E) shows increased GFAP mRNA expression. There is light GFAP mRNA expression in the corpus callosum at all ages. (F–I) A digoxygenin-labeled antisense GS probe was hybridized to representative 10-μm parasagittal sections from P0 (F, G) and P7 (H, I) forebrain. Glutamine synthetase (GS) mRNA is detectable in the SVZp at P0 (F) and P7 (H). However, GS mRNA expression is absent in the SVZa at P0 (G) and P7 (I), except for at the boundary between the SVZa and corpus callosum and next to the ependyma lining the ventricles (not shown in SVZa panels, but see F and



H). As with GFAP, GS mRNA is expressed throughout the corpus callosum at both P0 and P7. The inset in G is a similar P0 SVZa section stained with nuclear fast red to demonstrate the densely packed cells in this region (see also Fig. 4). Abbreviations: A, anterior; a, SVZa; CC, corpus callosum; CTX, cerebral cortex; D, dorsal; LV, lateral ventricle; p, SVZp. Scale bars in B, F, G, H, and I are 50 μm; the scale bar in E, 200 μm, applies to the remaining panels.

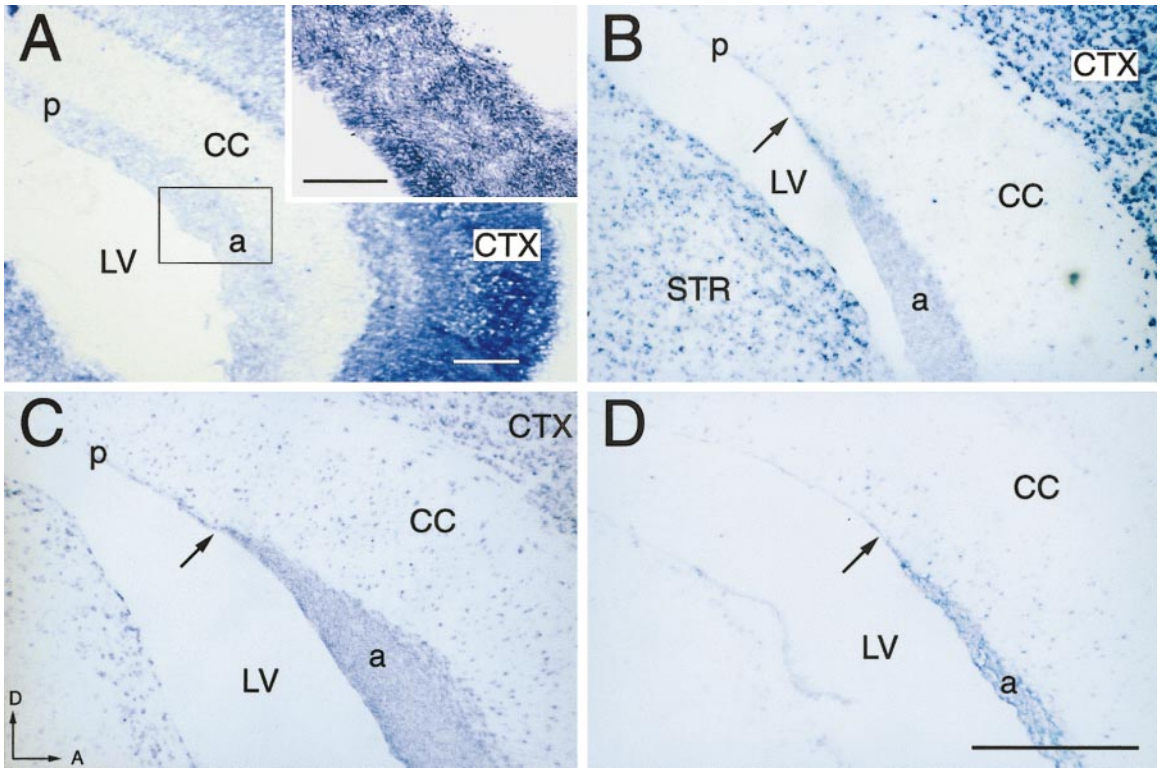
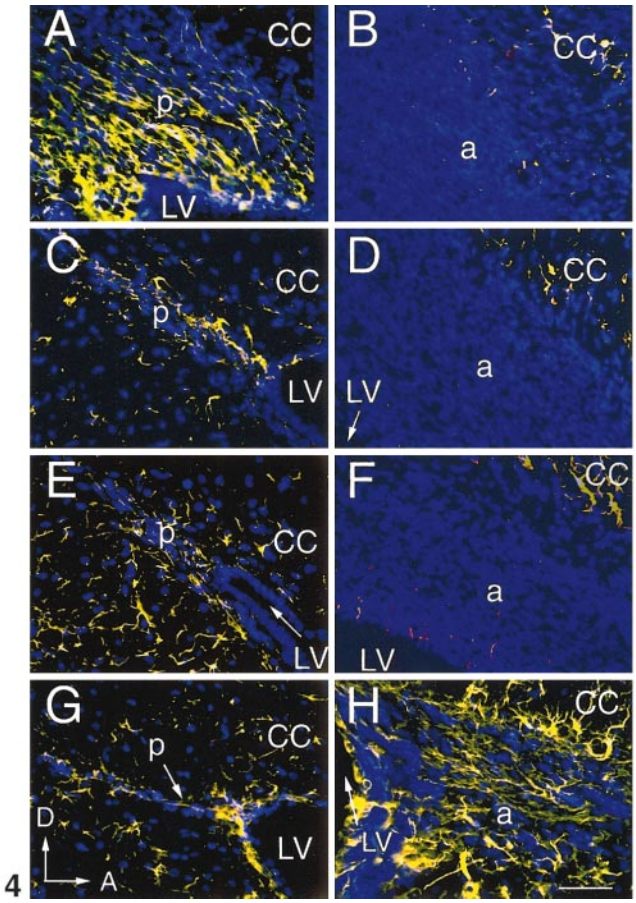


FIG. 3. Neonatal SVZa cells express neuron-specific Tα1 mRNA. (A–D) A digoxygenin-labeled antisense probe for the neuron-specific α-tubulin isoform, Tα1, was hybridized to representative 10-μm parasagittal sections from the forebrain of P1 (A), P7 (B), P14 (C), and P21 (D) animals. Tissue sections from P7, P14, and P21 animals used for these Tα1 *in situ* analyses are semiadjacent (within 100 μm) to those employed in the GFAP mRNA expression analyses in Fig. 2. In B–D the arrow approximates the transition between the SVZa and the SVZp. There is expression of Tα1 mRNA in the SVZa at all ages. The inset in A is a higher magnification view of the region of the SVZa indicated by the black box from a similar age-matched section. The P1 section in the inset was developed longer to more clearly demonstrate the cellular localization of the Tα1 mRNA *in situ* hybridization signal in the SVZa. At the three older ages observed, there is less Tα1 mRNA hybridization in the posterior SVZ. Decreasing expression of Tα1 mRNA during postnatal development is evident in the cerebral cortex and striatum (C, D). The identity of the cells expressing Tα1 mRNA in the corpus callosum is unclear but they may be late-migrating neurons destined for the cerebral cortex. The thickness of the subventricular zone decreases throughout postnatal development; this is particularly evident in the SVZp from P1 to P7. A reduction in the thickness of the SVZa, on the other hand, is not prominent until P21. Abbreviations: A, anterior; a, SVZa; CC, corpus callosum; CTX, cerebral cortex; D, dorsal; LV, lateral ventricle; p, SVZp; STR, striatum. The scale bar in A is 200 μm; inset of A is 100 μm; scale bar in D, 500 μm, applies to B–D.

and in the cerebral cortex overlying the minimally labeled corpus callosum (Fig. 3A and data not shown). A higher magnification view of a region of the P1 SVZa hybridized with the Tα1 antisense RNA probe is shown in the inset of Fig. 3A to demonstrate the expression of this gene within the tightly packed cells of the SVZa. Tα1 mRNA expression in the SVZa is specific, as evidenced by the absence of signal following hybridization with the corresponding sense probe (data not shown), but the intensity of the Tα1 mRNA signal in this region is typically less than in the neonatal cerebral cortex or striatum. Preliminary electron microscopic (EM) analysis of P7 SVZa supports the apparent homogeneity of cells in this region. All SVZa cells examined by EM appear to have morphological characteristics of immature and/or migrating neurons (data not shown). At P7, P14, and P21, the SVZa continues to express Tα1 mRNA, further supporting the suggestion that this progenitor cell population retains neuronal characteristics in the postnatal brain (Figs. 3B, 3C, and 3D). The light labeling of scattered cells in the corpus callosum may indicate the presence of migrating neurons destined for the cerebral cortex that were generated at an earlier time in the ventricular zone.

Immunohistochemical Detection of Cell-Type-Specific Proteins in the SVZ and RMS

To provide support for the functional significance of the differential cell type-specific gene expression detected in the SVZ, we analyzed the expression of GFAP (Fig. 4) and neuron-specific β-III tubulin (Fig. 5) with immunohistochemistry. The results of these analyses are consistent with our mRNA expression studies and demonstrate that from



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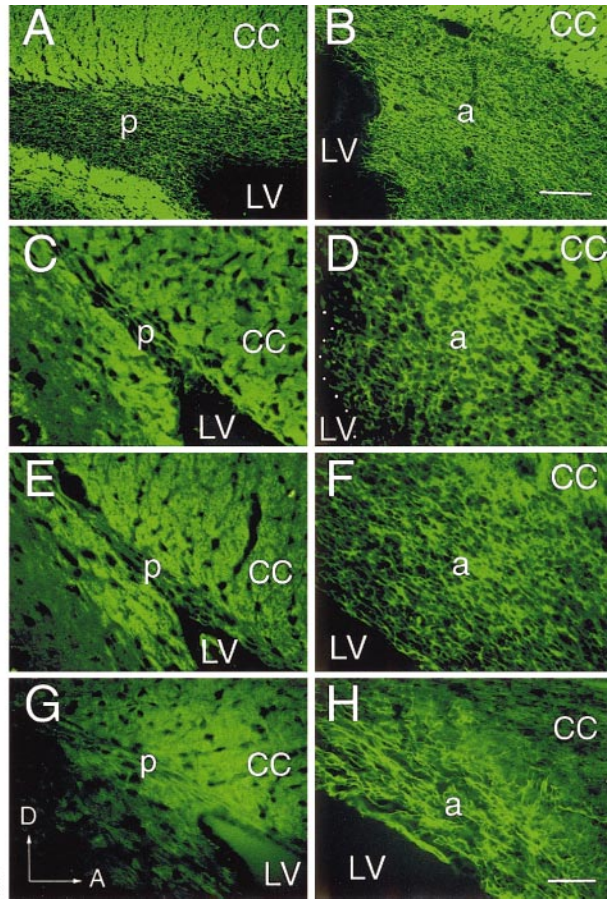

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FIG. 4. Spatio-temporal pattern of GFAP immunoreactivity in the postnatal telencephalic SVZ. (A–G) Fluorescent photomicrographs of representative 10-μm parasagittal sections of the SVZp (left column, A, C, E, and G) and SVZa (right column, B, D, F, and H) from P1 (A and B), P7 (C and D), P14 (E and F), and P21 (G and H) forebrain in which the cell nuclei are counterstained by DAPI (blue). The GFAP immunoreactivity appears yellow. Photomicrographs of SVZa and SVZp at each age were taken from the same section. While there is negligible GFAP immunoreactivity in the SVZa at P1 (B) and P7 (D), GFAP is apparent in the SVZa by P14 (F), but at lower levels than those of the overlying corpus callosum. By P21, the expression of GFAP protein in the SVZa is increased (H). The thickness of the SVZp decreases with postnatal development but intensely GFAP-immunoreactive cells are present in the SVZp at all ages studied. In the corpus callosum bordering the SVZa, GFAP immunoreactivity increases from P1 to P21, probably reflecting the maturation of astrocytic processes. Abbreviations: A, anterior; a, SVZa; CC, corpus callosum; D, dorsal; LV, lateral ventricle; p, SVZp. Scale bar in H, 50 μm, applies to all panels.

FIG. 5. Neuron-specific β-tubulin immunoreactivity is apparent in the SVZa at all postnatal ages examined. Fluorescent photomicrographs of 10-μm parasagittal sections of the SVZp (left column, A, C, E, and G) and SVZa (right column, B, D, F, and H) from P1 (A and B), P7 (C and D), P14 (E and F), and P21 (G and H) forebrains stained with the neuron-specific antibody TuJ1 (green). Intense TuJ1 immunoreactivity is evident in the SVZa at all ages examined (B, D, F, and H) and TuJ1 immunoreactivity in the SVZa is always stronger than in the age-matched SVZp (A, C, E, and G). The TuJ1 immunoreactivity observed in the corpus callosum most likely represents axonal fiber staining. The decrease in TuJ1 immunoreactivity seen in the corpus callosum at P21 reflects the down-regulation of this neuron-specific tubulin isoform in the mature brain. However, expression of this marker in nascent neurons remains strong in the P21 SVZa (see text). Abbreviations: A, anterior; a, SVZa; CC, corpus callosum; D, dorsal; LV, lateral ventricle; p, SVZp. The 50 μm scale bar in B also applies to A. The 100 μm scale bar in H applies to all other panels.

P1 through P7, GFAP is essentially absent within the rat SVZa (Figs. 4B, 4D, and 4F). Conversely, neuron-specific β-III tubulin is expressed in the SVZa throughout development, always more strongly than in the SVZp (Fig. 5). These data provide additional support for the contention that the SVZa is predominantly or exclusively neurogenic. The density of TuJ1-positive cells is apparently lower in the SVZp compared to the SVZa. Some of the staining in the SVZp may reflect the ongoing migration of prenatally generated neurons toward the cerebral cortex. However, we

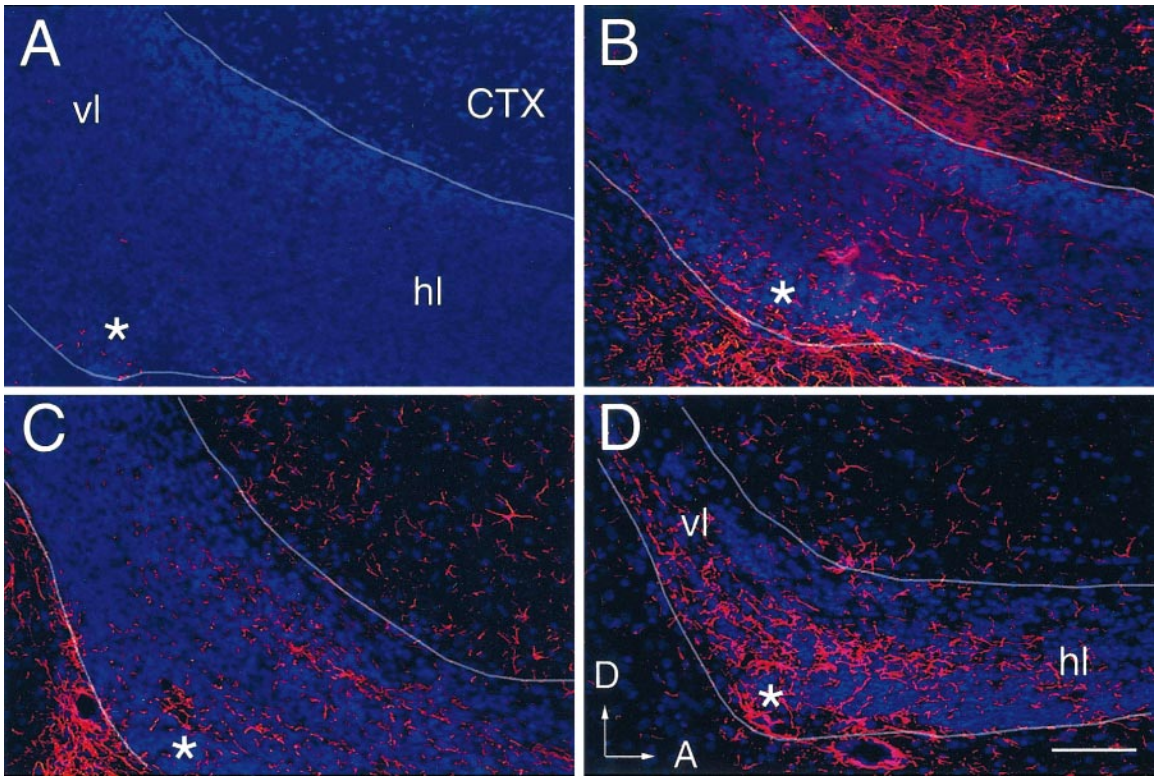

FIG. 6. Spatio-temporal expression of GFAP in the rostral migratory stream (RMS). (A–D) Fluorescent photomicrographs of representative 10-μm parasagittal sections through the RMS of the forebrain at P1 (A), P7 (B), P14 (C), and P21 (D). In each section, the cell nuclei are counterstained by DAPI (blue) and the astrocytes visualized with an antibody to GFAP (red). In A–D, the RMS represents that anterior extension of the SVZa shown in the same sections as in Fig. 4. At P1 (A), the sparse GFAP immunoreactivity is concentrated at the elbow of the RMS (asterisk), but absent from the SVZa (not shown). The expression of GFAP is increased around the elbow (asterisk) and in the horizontal limb of the RMS at P7 (B) and P14 (C). Note that at P7 (B), the GFAP fibers in the horizontal limb extend mainly perpendicular to the RMS, while at P14 (C), they run tangentially. By P21 (D), the tangentially oriented GFAP fibers are present in the horizontal as well as the vertical limb of the RMS. Most migrating SVZa-derived cells do not encounter GFAP-positive cells in the first postnatal week, indicating that astrocytic guidance is not necessary for the migration of these cells in the neonate. Abbreviations: A, anterior; CTX, cerebral cortex; D, dorsal; hl, horizontal limb of the rostral migratory stream; vl, vertical limb of the rostral migratory stream. Scale bar in D, 50 μm, applies to all ages.

cannot rule out a low, steady-state production of neurons in the SVZp. Collectively, the results substantiate the reports that the SVZp is predominantly, though not exclusively, gliogenic (Privat, 1975; LeVine and Goldman, 1988; Levison and Goldman, 1997), and that the SVZa is predominantly, and perhaps exclusively, neurogenic (Luskin, 1993; Menezes *et al.*, 1995).

To further examine the potential involvement of astrocytes in SVZa-derived cell migration, we have employed GFAP immunohistochemistry to determine the spatio-temporal appearance of GFAP in the rostral migratory stream (refer to Fig. 1A for orientation). GFAP is absent from the P1 SVZa (Fig. 4B) and most of the rostral-caudal extent of the P1 RMS (Fig. 6A), with the exception of a thin stream of sparsely distributed GFAP-positive cells situated in the center of the RMS (data not shown). There is also a small concentration of GFAP-positive cells located at the ventral border of the elbow of the RMS where the vertical and horizontal limbs come together (Fig. 6A). At P7, GFAP immunoreactivity is still not detectable in the SVZa but does appear within the RMS, mainly in the caudal part of the horizontal limb and in the subependyma of the olfactory bulb (Fig. 6B). In the center of the RMS, the GFAP-positive fibers are mostly oriented perpendicular to the direction of the migrating SVZa-derived cells, whereas at the periphery of the RMS the GFAP-positive fibers ensheath the migrating cells as described for the adult RMS by Lois *et al.* (1996). At P14, there is increased GFAP immunoreactivity in the RMS, and particularly in the horizontal limb, and at this stage most of the GFAP-positive fibers are oriented parallel to the pathway (Fig. 6C). GFAP immunoreactivity is more prevalent in the vertical limb of the RMS by P21 than at younger ages. However, the relative level of GFAP immunoreactivity is lower in the RMS adjacent to the SVZa

neuronal progenitor pool (not shown) and higher in the rostral-most migratory stream, distant from the SVZa (Fig. 6D and data not shown). The extent of GFAP immunoreactivity in the SVZa also increases by P21 (Fig. 4H). Thus, the developmental profile of GFAP expression suggests that at least for the first postnatal week, astroglial sheaths do not contribute to migratory guidance of SVZa-derived cells in the rostral migratory stream. Since the period of peak olfactory bulb interneuron production and migration occurs within the first 10 days of life (Altman, 1969), astroglial migratory guidance is not available to the majority of olfactory bulb interneurons. Our data do not directly address the possibilities that astrocytes provide migratory guidance to, or serve as a stem cell population for, adult SVZa-derived cells.

DISCUSSION

Previous studies employing nucleotide markers of cellular proliferation (Altman, 1969), retroviral lineage tracers (Luskin, 1993), and cell type-specific immunological markers (Kishi *et al.,* 1990; Menezes *et al.,* 1995) raised the possibility that the neonatal subventricular zone might be subdivided into a posterior, predominantly gliogenic region and an anterior neurogenic region. The cells arising in the anterior SVZ migrate through the RMS to the olfactory bulb and become fully differentiated, postmitotic interneurons. The present report further supports the suggestion that the SVZa constitutes a distinct region by demonstrating that cell type-specific gene expression can distinguish the anterior and posterior SVZ *in situ*. In particular, in the neonatal rodent brain, there is virtually no expression of astrocyte-specific mRNA or protein in the SVZa, whereas the vast majority of SVZa cells express neuron-specific proteins (Menezes *et al.,* 1995; Zigova *et al.,* 1998). In contrast, expression of these astrocyte-specific genes is readily apparent by birth in the posterior SVZ (Gates *et al.,* 1995). These observations, together with the prolific mitotic activity of the neonatal SVZa (Menezes *et al.,* 1995; Smith and Luskin, 1998), suggest that the neonatal SVZa is a homogeneous neuronal progenitor cell population.

Reverse transcriptase-PCR detection and evaluation of gene expression within the SVZa and other portions of the forebrain subventricular zone depend on obtaining a precise dissection of the SVZa separate from the remainder of the postnatal forebrain, especially the overlying, glial-rich corpus callosum. The efficiency and specificity of our RT-PCR analysis is demonstrated by the detection of GFAP mRNA from microdissected posterior SVZ and from neonatal cerebral cortex, but not from the microdissected SVZa (Fig. 1B). These results are also consistent with our immunohistochemical and *in situ* hybridization analysis of cell type-specific gene expression in these regions.

The cell type-specific markers employed in this study have been widely used to identify astrocytes and neurons. Glial fibrillary acidic protein mRNA and protein are routinely used as a specific markers for astrocytes (Zerlin *et al.,* 1995). Glutamine synthase, the other astroglial-specific gene employed in this study (Mearow *et al.,* 1989), is also expressed in ependymal cells lining the lateral ventricle. Nevertheless, our *in situ* hybridization analysis of GS mRNA expression supports the conclusion that the early postnatal SVZa is devoid of astrocytes although GS-positive cells are apparent at the border of the SVZa by P7. The S100-β gene has also been employed as an astroglial marker and is also expressed in ependymal cells (Zhang and McKanna, 1997). As with GFAP and GS, *in situ* hybridization for S100-β mRNA supports the conclusion that the early postnatal SVZa is devoid of astrocytes (data not shown).

A number of neuronal markers have been described including neuron-specific enolase (Schmechel *et al.,* 1980), neuronal nuclear protein NeuN (Mullen *et al.,* 1992), neuron-specific RNA splicing factors (e.g., the Hu proteins) (Okano and Darnell, 1997), and neuron-specific cytoskeletal proteins such as neurofilaments (Carden *et al.,* 1987) and tubulin isoforms (Bond and Farmer, 1993). The neuron-specific tubulin isoforms, including the α-tubulin transcript Tα1, and the neuron-specific β-III tubulin recognized by TuJ1, have been extensively employed and are among the earliest neuron-specific genes expressed in the developing nervous system (Miller *et al.,* 1987b; Easter *et al.,* 1993; Memberg and Hall, 1995). In the present study we have assessed Tα1 mRNA as well as both mRNA and protein expression for β-III tubulin to provide independent detection of the neuronal phenotype. Our studies are in agreement with previous reports (Jiang and Oblinger, 1992) of the expression of these neuron-specific isoforms throughout the neonatal rat brain, including the SVZa and RMS (Menezes *et al.,* 1995). The nascent neurons produced in the SVZa continue to express neuron-specific tubulin mRNA and protein at all postnatal ages examined. In the P21 cerebral cortex and striatum, however, we observed a decrease in the level of Tα1 mRNA and β-III tubulin protein. This is consistent with previous reports indicating that expression of both the Tα1 and the β-III neuron-specific tubulin isoforms in the brain is reduced following the second postnatal week of rodent development (Bond and Farmer, 1983; Bond *et al.,* 1984; Miller *et al.,* 1987a; Jiang and Oblinger, 1992).

The virtual absence of GFAP mRNA and astrocytes, as well as the tangential orientation of the RMS, suggests that migration of neonatal SVZa-derived cells to the olfactory bulb does not depend on glial guidance, unlike most immature, newly postmitotic CNS neurons in the developing telencephalon (Hatten, 1990; Kishi *et al.,* 1990; Rakic, 1990). Nevertheless, a specialized organization of GFAP-positive glial cells has been described in the adult RMS and it has been suggested both that these glia may influence migration of neurons destined for the olfactory bulb (Lois *et al.,* 1996) and that these cells are stem cells for SVZa-derived neurons (Doetsch *et al.,* 1999). It should also be noted that although the SVZa progenitor population is active throughout life (Bayer, 1983), the period for peak

olfactory bulb interneuron production is P0-P10 (Altman, 1969). In this period, our findings indicate that there is little to no astroglial-specific gene expression in the RMS and SVZa. Moreover, the first appearance of GFAP in the RMS is where the vertical and horizontal limbs come together, not where SVZa-derived cell migration originates. That astroglia are not required for migration of SVZ-derived cells is further supported by the recent report that neonatal SVZ-derived neurons are capable of rapid migration away from an SVZ explant cultured in a three-dimensional matrix, even in the absence of glia (Wichterle *et al.,* 1997). In addition, dissociated neonatal SVZa cells in primary culture are uniformly immunoreactive for TuJ1 but not GFAP, indicating that at this stage, the SVZa is devoid of astroglia (Luskin *et al.,* 1997).

Despite the absence of specific cells that may provide contact-mediated guidance in the neonatal RMS, the millions of neonatal SVZa-derived cells appear to be absolutely confined to migration within this pathway (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Lois *et al.,* 1996). Several molecular candidates may be involved in the restriction of SVZa-derived cells to the RMS. The polysialyated form of neural cell adhesion molecule (PSA-NCAM) is adhesive for presumptive olfactory bulb interneurons and this extracellular matrix molecule is enriched in the RMS (Tomasiewicz *et al.,* 1993; Cremer *et al.,* 1994). Moreover, the migration of SVZa-derived cells is attenuated in mice harboring null mutations of the NCAM gene (Tomasiewicz *et al.,* 1993). The extracellular matrix molecules tenascin and phosphacan have also been localized to the SVZa and RMS of the adult forebrain (Gates *et al.,* 1995). Alternatively, surrounding anti-adhesive or repulsive factors may delineate the RMS and prohibit exit of SVZa-derived cells from this rostral migratory stream. Recently, the roundabout ligand, slit, has been shown to be expressed by the overlying septum and to specifically repel migrating SVZa-derived neurons *in vitro* and *in vivo* (Wu *et al.,* 1999). Chondroitin sulfate proteoglycans, which, like slit, are inhibitory to neurite outgrowh, also surround the neonatal RMS and may mediate a repulsive function (Tomasiewicz and Luskin, unpublished findings).

These data do not rule out an important function for the GFAP-positive cellular processes investing the RMS in the adult rat (Lois *et al.,* 1996). For example, astrocytes in the adult RMS and SVZa may contribute to the continued segregation of SVZa progenitor cell progeny to the RMS and olfactory bulb in the mature brain. GFAP-positive astrocytes may also constitute the neuronal stem cell in the adult SVZa (Doetsch *et al.,* 1999). Nevertheless, our studies demonstrating the scarcity of GFAP immunoreactivity in both the SVZa and the RMS during the period of peak neurogenesis for olfactory bulb interneurons indicate that astrocytes are not required for either neurogenesis or cell migration by the neonatal SVZa.

Although GFAP mRNA is not detected in the neonatal SVZa, it is readily detected in RNA prepared from microdissected neonatal posterior SVZ, as expected. Compared with the SVZa, we also detect lower levels of expression of neuron-specific genes in the neonatal SVZp. Our RT-PCR and *in situ* hybridization analyses are in agreement in this regard and these data support the suggestion that the neonatal posterior SVZ is not a homogeneous population of glial progenitor cells (Levison and Goldman, 1993). Neuronal gene expression in the SVZp may result from earlier born neurons of the embryonic ventricular zone (the source of most forebrain neurons), still *en route* to a final destination within the cerebral cortex. Alternatively, neuron-specific gene expression in the neonatal posterior subventricular zone may indicate the presence of multipotential or even neuronal progenitor cells in this region. Several studies have documented the presence of multipotential cells surrounding the lateral ventricles adjacent to the striatum (Halliday and Cepko, 1992; Palmer *et al.,* 1995; Thomas *et al.,* 1996).

There are relatively few specific markers or genes that are available to distinguish subventricular zone progenitor cell populations in the postnatal CNS. Neuroepithelial markers such as nestin (Lendahl *et al.,* 1990) and vimentin (Schwob *et al.,* 1986) have been widely employed to distinguish mitotically active neuroepithelial cells from postmitotic differentiating neurons and glia. Such markers are of limited value in the postnatal subventricular zone, however, as this region is no longer a distinct epithelium and it is likely that active progenitor cells and differentiating progeny are intermixed in the SVZ. Nevertheless, SVZa-specific gene expression is likely to contribute to the unique phenotypic properties of this region. Several genes have been reported to be specifically localized to the postnatal subventricular zone including phosphacan and tenascin (Gates *et al.,* 1995), the platelet-derived growth factor receptor (Ellison *et al.,* 1996; Oumesmar *et al.,* 1997; Law *et al.,* 1998), and the putative transcription factor PBX-1 (Redmond *et al.,* 1996). However, the potential involvement of any of these gene products in the establishment or maintenance of the properties of SVZa neuronal progenitor cells is difficult to assess at the present time since the distinction between the SVZa and the remainder of the SVZ is usually not made. Future efforts will focus on the question of how the expression of specific genes contributes to the unique properties of SVZa neuronal progenitors.

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